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TECHNICAL MANUSCRIPT 175

FORMATION OF HEMOLYSIN BY STRAINS OF PSEUDOMONAS AERUGINOSA

FEBRUARY 1965

UNITED STATES ARMY
BIOLOGICAL LABORATORIES
FORT DETRICK

U.S. ARMY BIOLOGICAL LABORATORIES
Fort Detrick, Frederick, Maryland

TECHNICAL MANUSCRIPT 175

FORMATION OF HEMOLYSIN BY STRAINS OF *PSEUDOMONAS AERUGINOSA*

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Project 1A013001A91A

February 1965

ABSTRACT

Hemolysin is formed in sonic extracts of cells of *Pseudomonas aeruginosa* by the action of a heat-labile substance, probably an intracellular "release" enzyme, on a substrate from the disrupted cell. The substrate and most of the hemolysin released can be sedimented by high-speed centrifugation. Hemolysin-negative strains appear to possess no release enzyme but do contain the substrate, because addition of particulate matter to extracts of hemolysin-positive cells increases the rate and extent of hemolysin formation. The rate of hemolysin release in sonic extracts is strongly influenced by the concentration of the two reactants and minor dilution abolishes all activity. There is only a small amount of release enzyme and substrate present in 24-hour cells but increasingly greater amounts appear in extracts of 48- and 72-hour cells.

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I. INTRODUCTION

Although the hemolytic activity of strains of Pseudomonas aeruginosa has been widely recognized, little definitive work has been done on the production and characterization of the hemolysin. Berk[1,2] in preliminary studies has established that this hemolysin is a heat-stable substance produced in relatively low titer, that it can be concentrated by a variety of routine procedures, and that both extracellular and intracellular hemolysin can be detected. A survey has revealed that many species of pseudomonads are hemolysin producers.[3] The cited investigations employed the cellophane membrane technique for the production of hemolysin. Attempts to produce hemolysin by growth in liquid culture have failed.[1] This paper outlines the mechanism of production of hemolysin by strains of Pseudomonas aeruginosa and offers explanations for certain aspects of the formation of hemolysin that were not formerly understood.

II. MATERIALS AND METHODS

A. PSEUDOMONAS STRAINS

All the strains of Pseudomonas aeruginosa employed in this study were derived from clinical materials submitted for microbiological analysis to the Frederick Memorial Hospital, Frederick, Maryland. The identification of these isolates as P. aeruginosa was based on their reaction on triple sugar iron agar, their pigmentation, and their characteristic odor. Stock cultures were maintained at 4 C on Difco blood agar base slants and were transferred every three to four weeks to maintain viability.

B. HEMOLYSIN PRODUCTION ON MEMBRANE FILTERS

The method of producing hemolysin was essentially as described by Liu[3] and Berk.[1] Standard (0.45 μ) porosity membrane filters were placed on the surface of previously poured and solidified trypticase soy agar.* The membrane surface was inoculated by the spreading method with 0.1 ml of an overnight trypticase soy broth culture of the Pseudomonas strain to be investigated. After 48 to 72 hours of incubation at 37 C, the membranes were transferred to small beakers, where the microbial growth was washed off into 5 ml of 0.1 M phosphate buffer, pH 6.0, containing 0.35% sodium chloride (referred to as "buffer-saline"). This suspension was then centrifuged at 1640 x g in a clinical angle-head centrifuge for 10 min and the supernatant fluid containing the hemolysin was decanted and stored at 4 C until used. The packed cells were discarded.

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* Baltimore Biological Laboratories.

C. PREPARATION OF SONIC EXTRACTS

Plates of trypticase soy agar were inoculated by spreading a drop of an overnight broth culture of the *Pseudomonas* strain under study. After 48 hours of incubation, unless otherwise specified, the cells were washed off with 5 ml of 0.1 M phosphate buffer, pH 8.0, sedimented in an angle-head centrifuge, and resuspended in an equal volume of fresh buffer of the same composition. The suspended cells were then treated in a 9 kc sonic oscillator operating at maximum power output for 10 min. The resulting extract was then centrifuged in an angle-head clinical centrifuge for 15 min and the supernatant fluid was decanted and employed as described in Section III, Results.

D. ASSAY FOR HEMOLYSIN

Samples to be assayed were serially diluted in 1.5-fold dilution steps in the pH 6.0 buffer saline described above so that 0.5 ml of the dilution remained in each tube. Then 0.5 ml of a 1% suspension of washed sheep erythrocytes in 0.85% saline was added to each tube. The assay tubes were incubated in a 47 C water bath for 60 min. Subsequently each tube received 2 ml of 0.85% saline and the contents were centrifuged for 10 min to remove unlysed erythrocytes or erythrocyte debris. The supernatant fluid was decanted and the optical density at 550 mμ was determined in a Beckman Junior Spectrophotometer. The optical densities of the tubes were plotted on linear graph paper against the dilution factors of the tubes and a 50% hemolysis dilution was found graphically. The reciprocal of this dilution was designated as the number of hemolysis units per ml of the original sample. Replicate series of assays showed that the maximum error in this procedure was ±20%.

III. RESULTS

A. FACTORS AFFECTING FORMATION OF EXTRACELLULAR HEMOLYSIN

Some preliminary information about factors concerned with production of extracellular hemolysin by the membrane filter technique has already appeared.[4] It was found with a number of strains of *P. aeruginosa* that there was always considerable soluble protein in those preparations that contained hemolysin and it could be shown with a single strain that the amount of hemolysin was closely related to the amount of soluble protein present (Table 1). The report by Berk[1] that the yield of hemolysin was affected by the composition of the medium or by the amount of glucose in the medium has been confirmed in this laboratory. The increases in hemolysin yield have been small but definite and the effects of medium or glucose variation can always be detected. The specific activities (units of hemolysin per mg of protein) of such preparations are always nearly identical, suggesting that there is no qualitative change in the mechanism of formation of the hemolysin. The significance of the small variation in specific activities is not known; however, it is clear that there are no major changes in specific activity of hemolysin produced under a variety of conditions. A general literature survey[1,4] of hemolysin formation showed that there is little hemolysin produced in 24 hours of incubation but, after 48 and 72 hours' incubation, the hemolytic activity of membrane preparations is considerable. These facts, together with the observation[4] that hemolytically active preparations contain appreciable quantities of DNA, suggest that hemolysin arises as a consequence of cell autolysis. In the present work, changes in the temperature of incubation did not increase the production of hemolysin nor was the specific activity of the preparations altered by different incubation temperatures.

TABLE 1. EFFECT OF MEDIUM COMPOSITION OR GLUCOSE CONTENT ON HEMOLYSIN PRODUCTION BY A STRAIN OF PSEUDOMONAS AERUGINOSA

Medium[a/]	Hemolysin, units/ml	Protein, μg/ml	Specific Activity[b/]
TSA	2.1	130	16.1
TSA + 0.25% glucose	2.1	140	15.0
TSA + 0.05% glucose	2.6	180	14.4
TSA + 0.75% glucose	3.6	195	18.5
TSA + 1.0% glucose	4.1	225	18.2
BAB	3.6	320	11.2
Albimi agar	8.8	615	11.1
Tryptose agar	3.3	330	10.0
Casamino acids agar	trace	40	-
Heart infusion agar	2.6	213	12.2

a. TSA = trypticase soy agar; BAB = Blood agar base. Casamino acids agar is that medium described by Mennigman.[5]

b. The specific activity of the preparation is described as the hemolytic units per mg protein.

B. HEMOLYSIN IN CELL-FREE EXTRACTS PREPARED BY SONIC OSCILLATION

Cell rupture in a French pressure cell reportedly releases intracellular hemolysin from a strain of P. aeruginosa[3] and this intracellular hemolysin has properties very similar to those of the hemolysin in membrane filter preparations. Exposure of cells of a variety of strains of P. aeruginosa to sonic oscillation releases hemolysin into the surrounding medium. The results with a single strain are presented in Table 2. The high zero-time content of hemolysin arises from autolysis occurring on the membrane during growth. It is apparent that more hemolysin is released as the length of exposure to sonic oscillation increases. The specific activity of these preparations was variable but always considerably lower than the specific activity of extracellular hemolysin prepared by the membrane method.

TABLE 2. RELEASE OF HEMOLYSIN FROM CELLS OF PSEUDOMONAS AERUGINOSA BY DISRUPTION IN A SONIC OSCILLATOR[a]

Minutes of Oscillation	Hemolysin, units/ml	Protein, μg/ml	Specific Activity
0	7.1	490	14.5
5	10.0	890	11.2
10	11.0	1190	9.2
20	15.5	1780	8.7
30	16.5	1880	8.7

a. Cells of strain A grown by membrane filter method for 48 hr at 37 C. Harvested in 0.067 M phosphate buffer pH 6.0 and disrupted by treatment in the sonic oscillator at full output for the times indicated. Samples centrifuged in angle-head centrifuge for 10 min and supernatant fluid decanted and assayed for hemolysin and protein content.

C. APPARENT ENZYMATIC RELEASE OF HEMOLYSIN DURING INCUBATION OF SONIC EXTRACT

The differences in specific activity between extracellular and intracellular hemolysin plus other empirical considerations led to the concept that the hemolysin was being released either by an enzyme attacking a substrate exposed by disrupting the cell by sonic oscillation or by natural autolysis. Therefore, sonic extracts from 48-hr cultures of several strains were prepared as described and incubated at 37 C. At zero time and at selected intervals, a sample was withdrawn and heated for 10 min in a boiling water bath to inactivate the proposed enzyme. As controls, a large sample of sonic extract was heat-inactivated as described immediately after preparation and then incubated at 37 C, as were the other samples. The results (Fig. 1) show that there is a heat-sensitive release of hemolysin during incubation of sonic extracts and that there is no detectable hemolysin in the sonic extract of strains A or C immediately after preparation. Heat treatment immediately after preparation of the sonic extract abolishes the release of hemolysin during subsequent incubation at 37 C. Strain D of *P. aeruginosa*, which does not produce hemolysin by the membrane method, also did not yield any hemolysin during incubation of a sonic extract prepared from it.

The rate of release of hemolysin during incubation of sonic extracts at 37 C is strongly influenced by minor dilution. A sonic extract of strain A prepared from double the usual cell concentration was diluted as presented in Fig. 2 and the rate of release of hemolysin from these dilutions was determined. It is apparent that dilution by a factor of 2 lowers the rate of release of hemolysin by a factor of 5.2. Such data strongly support the concept that a release enzyme acts upon a substrate to release the hemolytic substance. Alternatively, these data could indicate that two enzymes are involved in the release of hemolysin. Such a system would require that the substrate be at saturation levels but the release of hemolysin from added particulate material (Fig. 3) indicates that saturation levels of substrate do not occur in these sonic extracts. Therefore, the simpler interpretation of one enzyme - one substrate reaction seems to be valid.

D. RELEASE OF HEMOLYSIN FROM VARIOUS STRAINS BY A SINGLE RELEASE ENZYME

Preliminary experiments have determined that the substrate acted upon by the release enzyme was particulate and could be sedimented by centrifugation. Accordingly, sonic extracts of several strains were prepared as described and immediately heated for 10 min in a boiling water bath to inactivate the release enzyme in the extract. The particulate material was sedimented by centrifugation at 28,000 x g for 5 hr and resuspended in one-half the amount of fresh 0.1 M phosphate buffer, pH 8.0. Equal amounts of these particles were then added to samples of freshly prepared sonic extract of strain A. The mixtures were then incubated at 37 C and samples were taken immediately and at appropriate intervals and heated in a boiling water bath for 10 min to inactivate the release enzyme of the strain A extract. The results (Fig. 3) show that the release enzyme of strain A is able to act upon the particulate material from any of the strains tested. It is of particular interest to note that hemolysin is released from particulate material prepared from strain D, suggesting that this strain does not produce hemolysin because of a deficiency in release enzyme rather

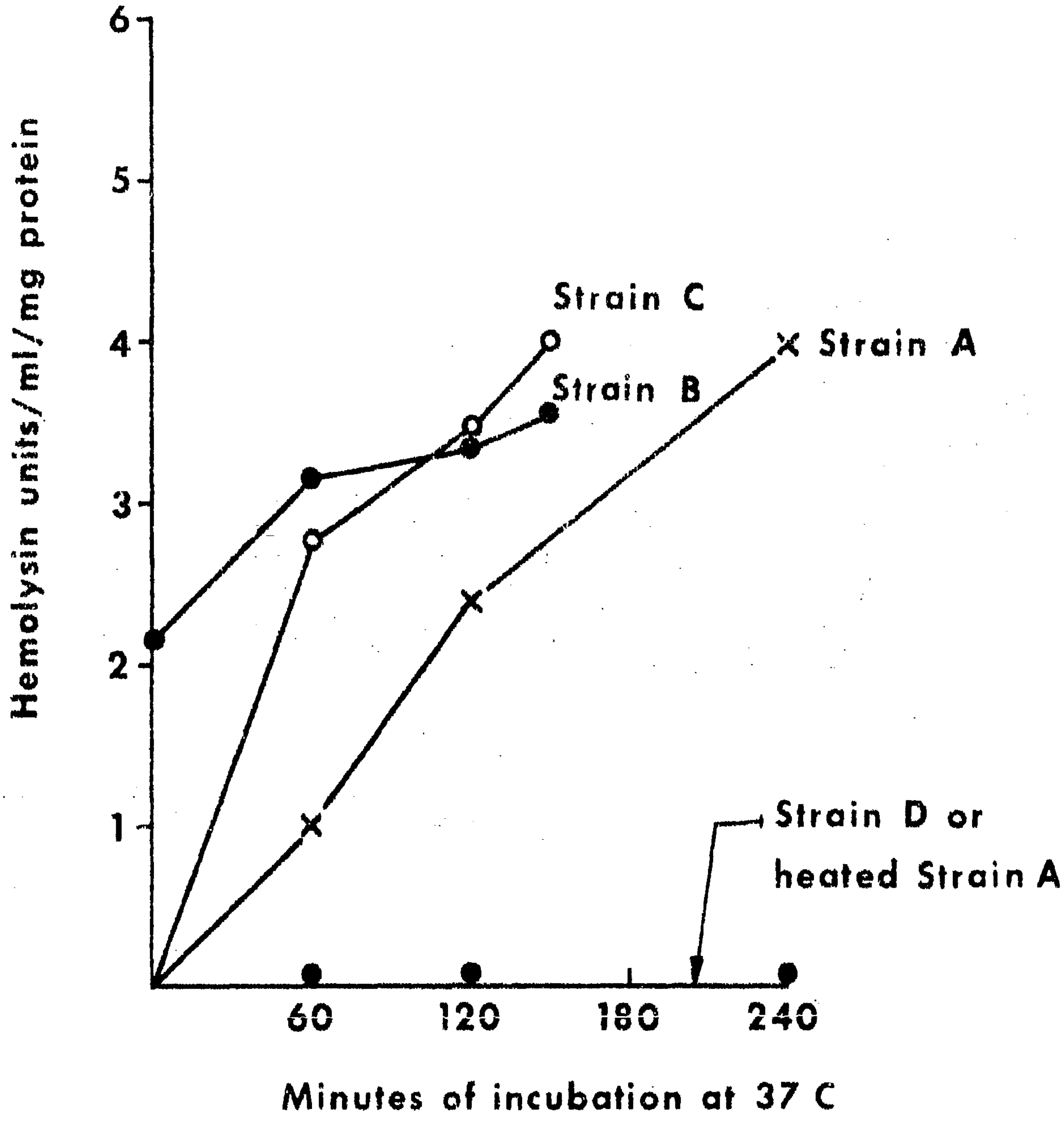

Figure 1. Release of Hemolysin by Incubation of Sonic Extracts of Several Strains of Pseudomonas aeruginosa.

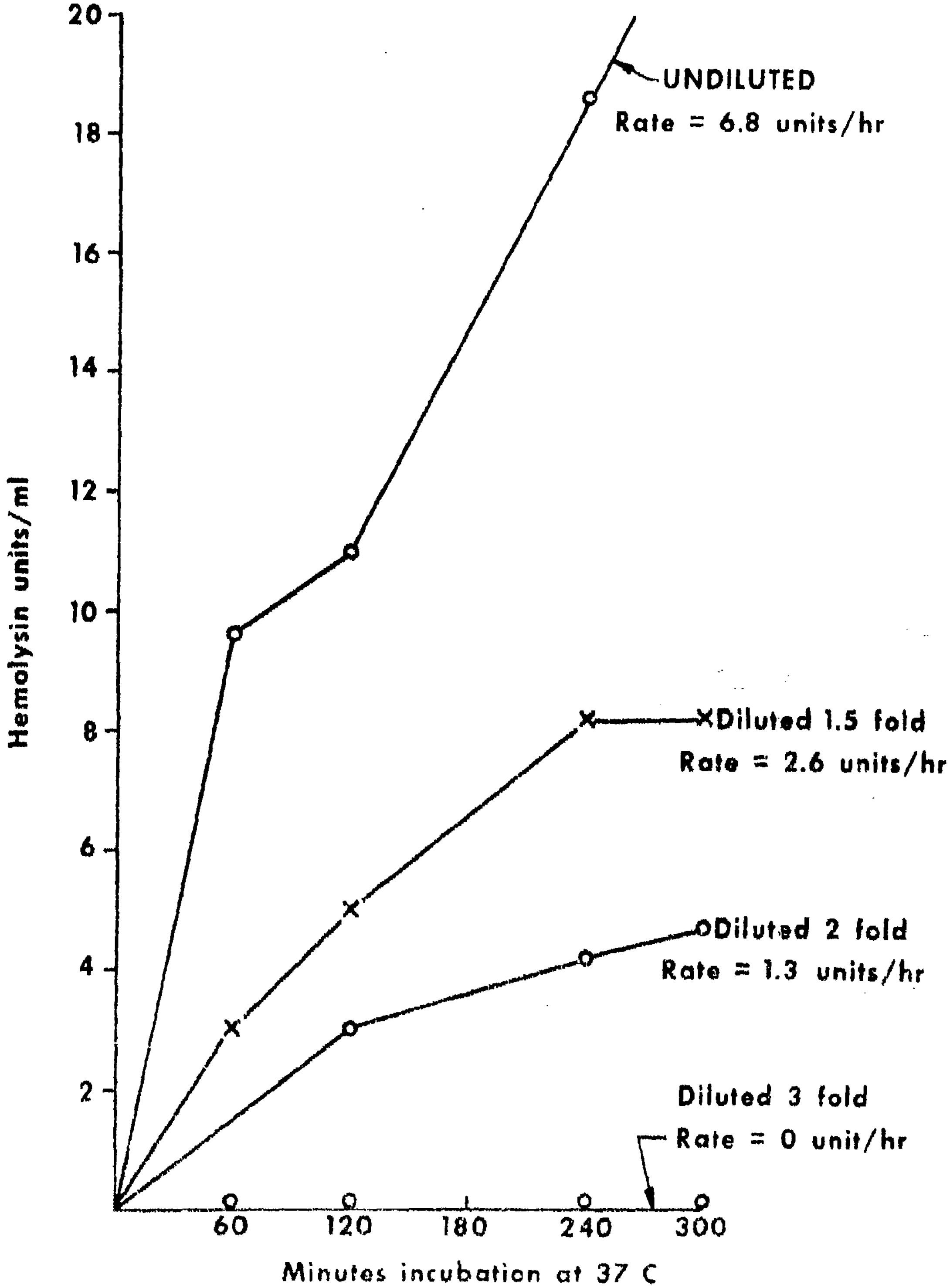

Figure 2. Effect of Dilution of Extract on Release of Hemolysin.

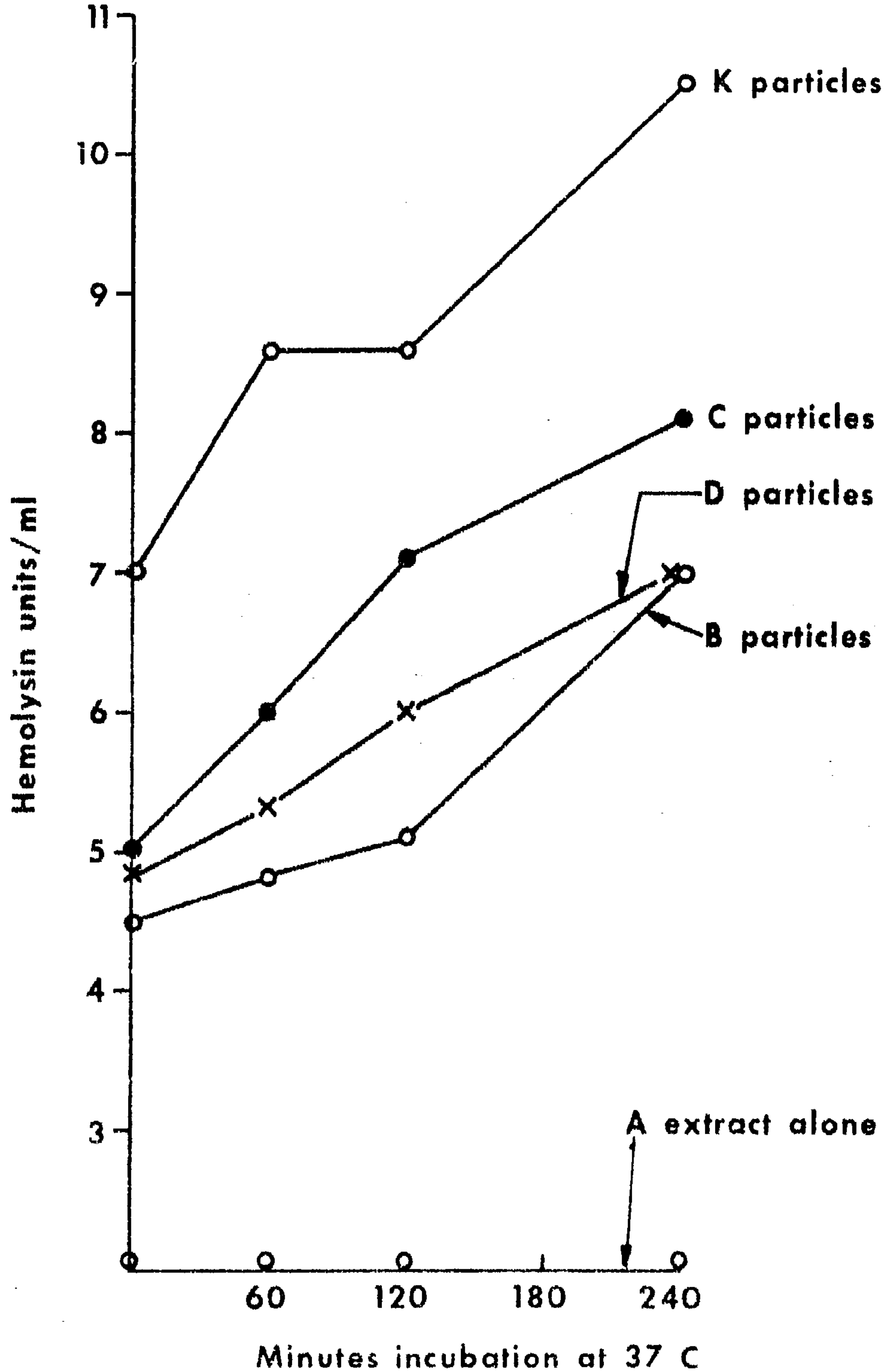

Figure 3. Release of Hemolysin from Particulate Material of Several Strains when added to Strain A Sonic Extract.

than in substrate. Other strains, hemolysin-negative by the usual criteria, have not been similarly tested. The hemolytic activity of the particulate matter before exposure to the release enzyme is always observed. It appears that sufficient agitation, such as in the preparation of these particle suspensions, leads to nonenzymatic hemolysin release.

E. HEMOLYSIN RELEASE IN SONIC EXTRACTS OF CELLS OF VARIOUS AGES

Since, by the membrane method, hemolysin is produced only after incubation for 48 hr or more and is associated with autolysis, it was of interest to determine the hemolysin release properties of sonic extracts prepared from cells of strain A grown for varying periods of time. Although the amount of protein in the extracts varied considerably, a comparison can easily be made by converting all results to the level of 2 mg protein per ml. This can be done taking into account the demonstrated influence of dilution on the release rate. The results presented in Fig. 4 show that 24-hr cells possess little of the release enzyme, as evidenced by the slow rate of liberation of hemolysin, and also possess little of the substrate, as demonstrated by the low final level of hemolysin attained. In contrast, the 48-hr and 72-hr cells possess much more enzyme and substrate, the 72-hr cells in particular possess both reactants in large amount.

F. PARTICULATE NATURE OF THE HEMOLYSIN

It was assumed, in consonance with other hemolysins studied, that this hemolysin was a protein molecule with unusual heat stability.[1] Several attempts to band the hemolysin by sucrose density gradient centrifugation were unsuccessful and all the hemolytic activity could be recovered in the pellet that developed in the bottom of the tube. Therefore, the sedimentability of the hemolysin during release from substrate in a sonic extract of strain A was determined by centrifugation at 28,000 x g for 5 hr. The results presented in Table 3 illustrate the particulate nature of the hemolysin but also show that, as the incubation of sonic extract proceeded, some hemolysin was released that was not sedimentable and may be in a truly soluble form. It is of interest that the "soluble" hemolysin never exceeds 25% of the total hemolysin and that release of the soluble form stops when total release also ceases. Included in Table 3 are the results obtained from centrifuging a sample of extracellular hemolysin produced by the membrane method. It is apparent that this sample, also, possesses mostly sedimentable hemolysin but that some hemolysin remains in the supernatant fluid. The suggestion[1,2] that hemolysin produced by growth on membrane is qualitatively different from the hemolysin released by cell rupture is not supported by this observation.

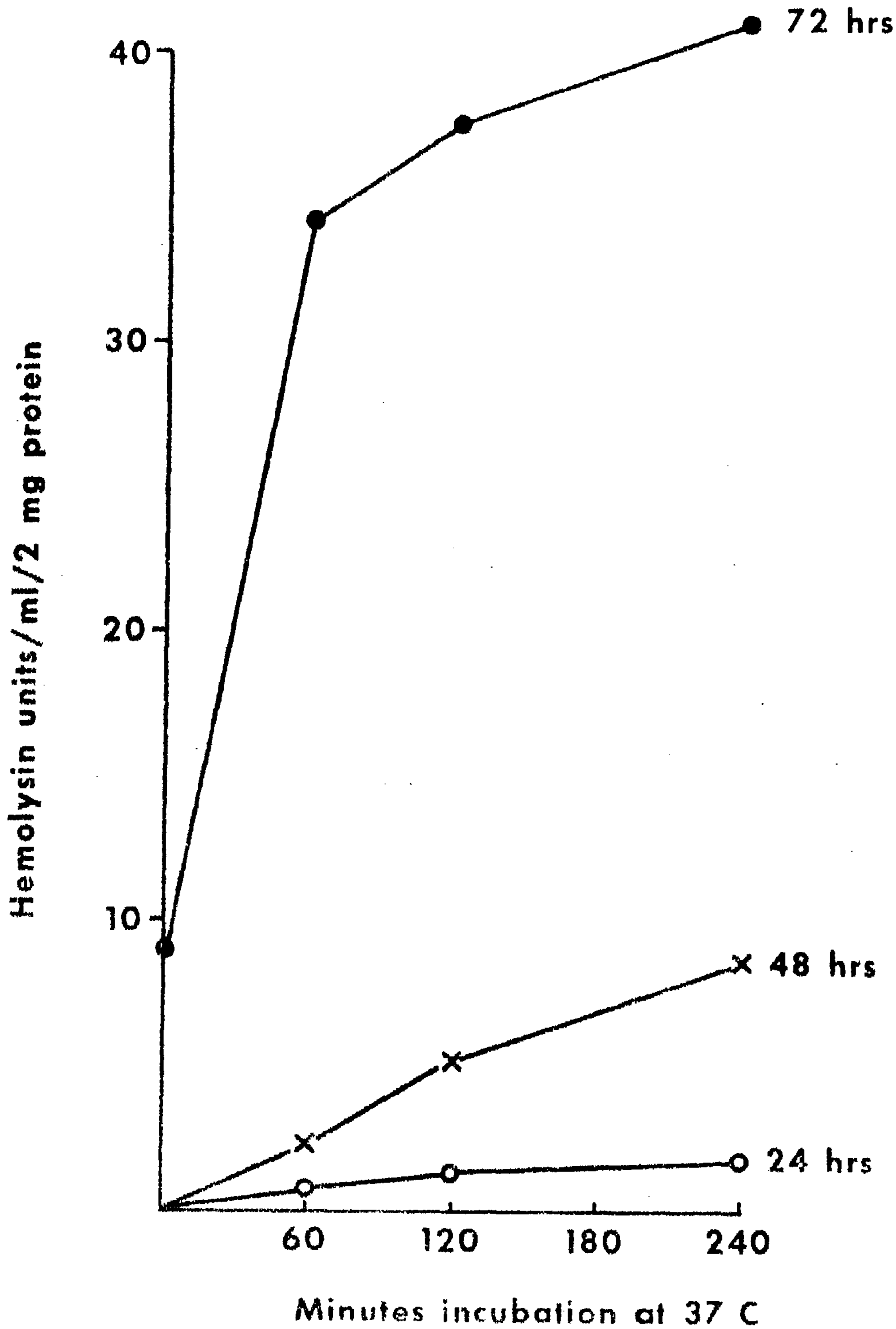

Figure 4. Release of Hemolysin from Sonic Extracts Prepared from Cells of Strain A of Various Ages.

TABLE 3. SEDIMENTABILITY OF THE HEMOLYSIN RELEASE BY INCUBATION OF SONIC EXTRACTS OF STRAIN A OF PSEUDOMONAS AERUGINOSA

Incubation, minutes	Total Hemolysin Units	Hemolysin Units in Supernatant
0	0	0
30	3.1	0
60	4.7	1.2
120	6.8	2.0
240	10.5	2.4
300	11.5	2.7
Extracellular preparation	5.8	1.9

IV. DISCUSSION

The results demonstrating the influence of concentration of sonic extract on the rate and extent of hemolysin liberation suggest the reason for failure to obtain hemolysin in liquid cultures.[1] Autolysis of cells in liquid culture leads to such dilution of the cell debris and intracellular contents that hemolysin is not liberated by the enzyme at an appreciable rate. Obviously, during cultivation on solid medium, the cell concentration is much higher and there is little or no dilution of the autolytic products, so that hemolysin production follows autolysis. In addition, the higher specific activity of hemolysin preparations obtained by growth on membrane as compared with sonic extracts probably results from the relatively prolonged incubation time for substrate and release enzyme on the membrane filter.

At present, nothing is known of the precise nature of the hemolysin or the release enzyme that liberates it from the particulate material of a sonic extract. The release enzyme is probably not concerned directly with the autolytic process itself, since several strains autolyse extensively but produce no hemolysin. The particulate material from one of these strains (D) contains hemolysin that can be liberated by treatment with strain A sonic extract (Fig. 3). However, as the culture ages, the cells possess more release enzyme and more substrate than in young cultures so that upon autolysis, conditions are favorable for liberation of hemolysin. The fact that most of the hemolysin is sedimentable and must be composed of particles larger than 100S suggests that fragments of the cell wall or cell membrane carry the hemolysin and express hemolytic activity. Such observations have very recently been made with cell wall fractions of mycobacteria.[8] To what extent the hemolytic activity of other members of the Enterobacteriaceae is similarly produced is unknown.

Attempts to isolate and identify the release enzyme and the nonsedimentable form of the hemolysin are in progress. Literature reports[1] and unpublished work in this laboratory attest to the salting out of the hemolysin upon addition of ammonium sulfate. We have found that these procedures give erratic and unsatisfactory results. In view of the demonstrated particulate nature of the bulk of the hemolysin, the unreliability of salting-out manipulations becomes clear because the amount of hemolysin collected by centrifugation following addition of ammonium sulfate depends on the size of the hemolytic particles and on the speed and time of centrifugation. The probable heterogeneity of the hemolytic particle sizes would preclude the employment of customary techniques for the isolation of a single molecular or particulate species. A recent paper by Berk[7] details the problems encountered in attempting to purify the hemolysin from a preparation that must be largely particulate.

Finally, it appears that this hemolysin plays only a minor role in the pathogenicity and/or virulence of this organism. The only pathological process that might meet the requirements for hemolysin formation (high-cell density and extensive autolysis), is an abscess. However, the recent report[8] that colicine can be potentiated and endotoxin altered by treatment with hemoglobin renews the expectation that the ability to produce hemolysin is related to the pathogenicity of enteric bacteria.

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